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Monitoring Cyclosporin A (Ciclosporin, INN) Concentrations in Whole Blood: Evaluation of the EMIT™ Assay in Comparison with HPLC and RIA

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Summary: We report here on an evaluation of the enzyme-multiplied immunoassay technique (EMIT™) from Syva for cyclosporin A (ciclosporin, INN) concentration measurements in whole blood. The assay incorporates a monoclonal antibody for specific determination of ciclosporin. Measurements by EMIT were performed on the Cobas Mira-S from Roche. A total of 197 blood specimens from heart- (n = 74), kidney- (n = 62) and liver- (n = 61) transplant recipients were analyzed. EMIT values correlated well with those obtained by HPLC, as well as with those obtained by a selective radioimmunoassay (INCStar). Ciclosporin concentrations determined by EMIT (y) agreed very well with those by RIA, and averaged 8% higher than those by HPLC (x) [n = 197, $\bar{x}$ = 143 µg/l, $\bar{y}$ = 155 µg/l, y = 1.09x − 0.6, r = 0.969]. Within-series and between-days CVs ranged from 4.6% to 7.3% for ciclosporin concentrations > 100 µg/l, and from 5.5% to 10.5% for ciclosporin concentrations between 69.5 µg/l and 100 µg/l. The within-series CV for a concentration of 45.5 µg/l was 14.8%. Calibration employing a 2-point mode instead of a continuous mode of UV-signal evaluation improved the precision of the EMIT assay at low ciclosporin concentrations. Sample pretreatment required thorough and skilful performance to avoid false positive ciclosporin measurements. We conclude that the EMIT assay is specific, and rapid to perform. It can be effectively used in the monitoring of ciclosporin concentrations in whole blood.

Introduction

Routine monitoring of the cyclosporin (ciclosporin, INN) concentration in whole blood has been recommended for guiding individualized adjustment of the ciclosporin dosage for adequate immunosuppression and to reduce toxicity (1–3). Different immunoassays and high performance liquid chromatography (HPLC) have been used for monitoring ciclosporin concentrations in whole blood. Proficiency tests, external surveys, and method comparisons have shown that the variety of the immunoassays employed, as well as the different HPLC methods, led to insufficient comparability between laboratories (4–6). HPLC is a method that enables in principle selective separation of ciclosporin from its metabolites (7). Nonetheless, despite the superior specificity of ciclosporin measurements by HPLC there are analytical reasons that argue against this technique for regular routine monitoring. The complexity of HPLC methods for ciclosporin analyses in blood requires the skill of a well-trained operator to avoid poor reproducibility of the results obtained (8). Furthermore, the long turnaround time due to sample pretreatment and chromatography is unfavourable for regular ciclosporin monitoring with larger series of samples. The development of selective immunoassays for determination of the parent drug is complicated by the extensive metabolism of ciclosporin. The availability of radio-

immunoassays (RIA) involving a selective monoclonal antibody has improved selective ciclosporin analysis. Cross-reactivities with certain ciclosporin metabolites are minor even when samples contain large amounts of ciclosporin metabolites (9, 10). The introduction of a fluorescence polarization immunoassay (FPIA) employing a monoclonal antibody has made accessible relatively specific ciclosporin measurements by non-isotopic immunoassay technology (11). The major advantage of this semi-automated procedure, run on the TDx (Abbott), is an easy-to-perform sample pretreatment, and improved management of large numbers of samples. The reproducibility of ciclosporin determinations is good. Recently, a specific EMIT for ciclosporin analysis in whole blood using semi-automated feasibility has been introduced by Syva Company (Palo Alto, CA). We report here our evaluation of this assay, which was run on the Cobas Mira-S (Roche). Method comparisons were performed using HPLC and the RIA from INCStar which has been the in-house routine method for ciclosporin monitoring. The first goal was to evaluate the specificity of the EMIT by correlation studies with specimens from transplant recipients. Furthermore, we investigated modifications of the sample pretreatment and the instrument settings in order to improve the analytical performance of the EMIT assay for ciclosporin.

Materials and Methods

EMIT assay

The EMIT™ ciclosporin assay kit was provided by Syva Co. (Darmstadt, Germany). Reagent A contains NAD^+, the substrate glucose-6-phosphate, and a monoclonal antibody selective for ciclosporin. Reagent B contains ciclosporin linked to glucose-6-phosphate dehydrogenase enzyme (EC 1.1.1.49), and Tris buffer. The test kit is complemented by a diluent (consisting of Tris buffer, surfactant, and preservatives), and six whole-blood calibrators (ciclosporin concentrations: 0, 50, 100, 200, 350, and 500 µg/l). Methanol p.a. grade was purchased from Merck (Darmstadt, Germany). At the outset of our study, sample pretreatment was performed according to Syva's original assay protocol. The original protocol prescribes pipetting of 100 µl whole blood from patient specimens, calibrators, and control materials into round bottom polypropylene tubes. A pipettor operating with positive displacement and with a glass capillary (Transferpettor, Rudolf Brand GmbH, Wertheim, Germany) was used for the pipetting of whole blood. Proteins were precipitated by the addition of 200 µl methanol followed by 1–2 min of incubation. Vigorous centrifugation (11 000 min^{-1}; 10 500 *g*) for 5 min was performed using a desk centrifuge from Abbott. We modified the sample pretreatment after it became obvious that the recommended procedure was inappropriate for separating the supernatant easily from floating particles. One modification was to transfer the supernatant after centrifugation into a clean conical polypropylene tube and repeat the centrifugation. The other, less labour intensive modification, recommended later in a revised Syva protocol, doubled the volume of the sample (to 200 µl) and that of the methanol (to 400 µl), but with only one centrifugation step. An aliquot (100 µl) of the supernatant was diluted with 200 µl diluent using a pipetter/dilutor from Syva Co. The mixture was transferred into polypropylene sample cups, and the EMIT assay was performed on a Cobas Mira-S analyzer (Roche Analytical Instruments; Basel, Switzerland). During the study the instrument was operated exclusively for ciclosporin analyses. The enzyme activity was monitored in a continuous mode according to Syva's prescription for the assay. The instrument was programmed to monitor the absorbance of NADH at 340 nm for 350 seconds, thereby recording 15 absorbance measurements at intervals of 25 s. The calculated mean absorbance difference, to the ciclosporin related concentration, was the average of four absorbance differences measured between points 11 and 15 (fig. 1). Each run was recalibrated. We also investigated a modification of the measuring design employing a 2-point mode instead of measurements in the original continuous mode. The measuring time was prolonged to 400 s, and the absorbance difference was monitored between measuring points 13 and 17 (fig. 1). The lower limit of the measuring range for ciclosporin concentrations was set at 50 µg/l according to Syva's recommendation, even though lower ciclosporin concentrations were calculated and recorded by the instrument.

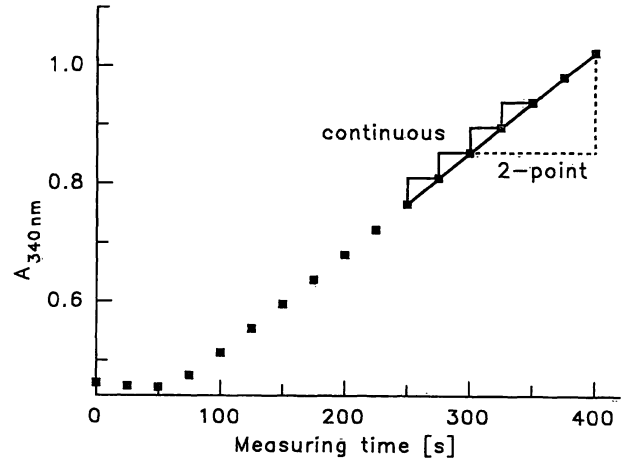


Fig. 1. Two measuring designs for NADH absorbance monitoring at 340 nm: a continuous mode (⌐¯) employing a measuring time of four intervals of 25 s, and a 2-point mode (····) employing an interval of 100 s.

RIA routine method

The RIA (CYCLO-Trac® SP-whole-blood radioimmunoassay, INCStar Corp., Stillwater, MN) was performed according to the manufacturer's instructions. Calibrators were prepared in-house employing whole blood from donors, which was supplemented with ciclosporin standard material in methanol solution (provided by Sandoz, Basel, Switzerland).

HPLC method

High performance liquid chromatography was performed on an HP-1090 instrument (Hewlett Packard, Waldbronn, Germany). Sample pretreatment was performed by several liquid-liquid extraction procedures prior to HPLC analysis. Sequential reversed-phase chromatography was run with coupled columns: Column-1 contained in-house filled packing material C_6 (Spherisorb; Phase Separations Inc., Norwalk, CT), and column-2 contained C_{18} (Hypersil-ODS; Shandon Scientific Ltd., Cheshire, U.K.). We eluted ciclosporin from the columns by use of a gradient consisting of water, methanol and acetonitril. Calibrators were prepared in-house as previously described (6).

Control specimens

Three whole blood ciclosporin controls (levels 1 – 3) were purchased from Bio-Rad (München, Germany). Different control materials prepared in-house had ciclosporin concentrations of 50, 100, and 250 µg/l. One set of control specimens was provided by Syva Co. The set consisted of 10 specimens supplemented with ciclosporin at concentrations between 60 µg/l and 425 µg/l. This set was used for analytical recovery experiments of ciclosporin. The limited amount of sample volume provided allowed duplicate measurements with EMIT, but only single analysis with HPLC.

Clinical specimens

Blood specimens were collected from patients during the post-transplant course with ciclosporin immunosuppression. Transplant recipients mostly received ciclosporin in combination with other immunosuppressants (12). All specimens were drawn 12 h after the last administration of a ciclosporin dose. Whole blood was collected into tubes coated with the anticoagulant ethylenediaminetetraacetic acid (EDTA). The first analysis was performed by RIA in the course of the regular ciclosporin monitoring. Analyses of the ciclosporin concentration by EMIT and by HPLC were performed not later than 5 days after collection of these specimens. The specimens had been stored at 4 °C. At most four specimens from the same patient were employed in each method comparison. The specimens were collected on different days.

Study design of method comparisons

We performed two method comparison studies with specimens from transplant recipients. Study I followed exactly the Syva assay protocol. We employed the sample pretreatment with 100 µl sample volume, and single centrifugation. Calibration and ciclosporin analysis were performed by signal evaluation employing the continuous mode. Study I included 208 specimens from 158 different patients. The aim of this study was the evaluation of the specificity of EMIT in comparison both with HPLC, and with specific RIA. Therefore, the selection of patient specimens was such that samples with relatively low ciclosporin metabolite concentrations (renal, heart), and those with larger metabolite concentrations (heart, liver) were chosen.

The aim of Study II was not primarily the evaluation of the specificity of EMIT; rather, we assessed the analytical performance of EMIT using two modifications of the assay. One modification of EMIT employed a small sample volume (100 µl), two centrifugation steps, and the continuous mode for signal evaluation (^{1}EMIT). The second modification of the assay was performed with a 200 µl sample volume, one centrifugation step, and measurements by the described 2-point mode (^{2}EMIT). Method comparisons in Study II were performed with 102 blood specimens obtained from 72 different renal transplant paediatric out-patients. Comparisons were performed with RIA, but not with HPLC. The comparisons EMIT versus RIA should provide sufficient information about the analytical performance of EMIT.

The method comparisons in Study I and Study II were statistically evaluated by standardized principal component analysis (13), and the paired *Student*'s t-test.

Results

Measuring design

The non-linear calibration curve of the EMIT had very low slopes on use of the continuous mode. The greatest recorded difference in absorbance range was less than 0.01 absorbance units per 25 s for the ciclosporin concentration of calibrator 6 (500 µg/l) at the upper end of the measuring range. Although absorbance differences of the ciclosporin measurements were averaged over readings of four consecutive 25 s intervals, random variation was relatively large. This observation was not unexpected, considering the specified reproducibility of 0.0009 absorbance units of the Cobas Mira-S instrument. The absorbance differences were greater, and the calibration curve was steeper, employing the 2-point calibration mode as described above. Statistical calculations revealed that

Tab.1. Study of the imprecision of the EMIT ciclosporin assay

Material	Ciclosporin (µg/l)	CV (%)	n	Analytical design
Within-series				
LEVEL-1*	69.5	8.0	20	Single centrifugation, continuous mode
LEVEL-1*	73.7	5.5	20	Double centrifugation, 2-point mode
H-100**	100.6	6.6	20	Double centrifugation, 2-point mode
LEVEL-2*	183.2	4.6	20	Single centrifugation, continuous mode
LEVEL-2*	209.7	4.6	20	Double centrifugation, 2-point mode
LEVEL-3*	322.6	5.1	20	Single centrifugation, continuous mode
H-400**	370.9	5.0	20	Double centrifugation, 2-point mode
Between-days				
H-50**	45.5	14.8	29	Single centrifugation, continuous mode
LEVEL-1*	75.9	10.5	29	Single centrifugation, continuous mode
H-100	92.6	7.5	29	Single centrifugation, continuous mode
LEVEL-2*	190.1	7.2	29	Single centrifugation, continuous mode
H-250**	241.1	7.0	29	Single centrifugation, continuous mode
LEVEL-3*	340.2	7.3	29	Single centrifugation, continuous mode

* Control specimens from Bio-Rad
** In-house prepared control specimens

Tab. 2. Calibration cross-check of EMIT and HPLC using EMIT calibrators

Ciclosporin (µg/l)	Day I				Day II				Day III			
	EMIT Ciclosporin $\bar{x} \pm SD$ (µg/l)	Re-covery (%)	HPLC Ciclosporin (µg/l)	Re-covery (%)	EMIT Ciclosporin $\bar{x} \pm SD$ (µg/l)	Re-covery (%)	HPLC Ciclosporin (µg/l)	Re-covery (%)	EMIT Ciclosporin $\bar{x} \pm SD$ (µg/l)	Re-covery (%)	HPLC Ciclosporin (µg/l)	Re-covery (%)
C-0•	0◆		<25		4.3 ± 3.0◆		<25		0◆		<25	
C-50•	46.5 ± 7.3	(93)◆	42	(84)	62.0 ± 11.5	(124)◆	49	(98)	55.3 ± 11.8	(111)◆	53	(106)
C-100•	92.3 ± 7.1	(92)◆	85	(85)	103.5 ± 5.2	(104)◆	113	(113)	95.3 ± 3.5	(105)◆	83	(83)
C-200•	175.3 ± 6.6	(88)◆	190	(95)	194.3 ± 11.6	(97)◆	204	(102)	186.3 ± 2.2	(93)◆	187	(93)
C-350•	330.5 ± 13.6	(94)◆	336	(96)	361.8 ± 23.2	(103)◆	435	(124)	375.8 ± 14.1	(107)◆	323	(92)
C-500•	504.8 ± 35.4	(101)◆	509	(102)	558.5 ± 41.3	(112)◆	492	(98)	481.3 ± 20.8	(96)◆	489	(98)

• Nominal concentrations of EMIT calibrators
◆ EMIT mean concentrations, standard deviation (SD), and recovery calculated from 4 replicates

the single but 4-fold higher signal obtained by the 2-point mode, instead of the averaged but much smaller absorbance difference obtained by the continuous mode, should improve the precision of the assay at low ciclosporin concentrations.

Study of the imprecision of the EMIT™ ciclosporin assay

The within-series imprecision was investigated using two different analytical designs. One design employed single centrifugation during sample pretreatment, and recording the NADH absorbance by the continuous mode, as recommended by Syva. The alternative design employed double centrifugation, and a 2-point mode for the NADH absorbance recording. At ciclosporin concentrations of 101 µg/l up to 371 µg/l the coefficients of variation (CVs) within-series ranged between 6.6% and 4.6% regardless of the employed sample pretreatment mode and the UV-signal evaluation (tab. 1). We observed a CV of 8% for a ciclosporin concentration of 69.5 µg/l (LEVEL1) using the continuous mode. However, analyses of this control specimen utilizing the 2-point measurement design and two centrifugation steps determined a mean ciclosporin concentration of 73.7 µg/l and a CV of 5.5%. Evaluation of the between-days imprecision strictly followed the initial Syva protocol. At ciclosporin concentrations between 92.6 µg/l and 340 µg/l the CVs were around 7% (tab. 1). At ciclosporin concentrations between 75.9 µg/l and 45.5 µg/l the CVs were between 10.5% and 14.8%.

EMIT calibrator cross-check, and analytical recovery

The six EMIT calibrators were cross-checked by EMIT and by HPLC on three days during the study (tab. 2). The employed sample pretreatment and the measuring design for EMIT were performed according to the original Syva assay prescription. All calibrators were assayed in duplicate both with regard to the sample pretreatment, and to the measuring procedure. We observed satisfactory mean analytical recoveries between 88% and 112% with EMIT, except with calibrator C 50 on day-II where the analytical recovery was 124%. Multiple analysis by HPLC was not performed due to the limited volume of the specimens. The analytical recoveries obtained by the HPLC method ranged between 83% and 113%, except for one result which was 124% (C 350; day II). Regarding only single measurements per day the inaccuracy of HPLC was acceptable. The cross-check at all revealed no systematic calibration error in either of the two assays.

Analytical recovery of ciclosporin in supplemented whole-blood samples

Nine specimens provided by Syva Co. had ciclosporin target concentrations between 60 µg/l and 425 µg/l, one specimen was a blank (tab. 3). The employed sample pretreatment and the measuring design for EMIT were performed according to the original Syva assay prescription. Specimens were analyzed on two days by EMIT, but only once by HPLC due to the limited amount of specimen available. Determinations by EMIT revealed a mean recovery of 97% (range: 86–109%) on day I, and 101% (range: 93–113%) on day II. The analytical recovery obtained by HPLC ranged from 97% to 113% (mean: 103%).

Method comparisons of Study I

Eleven of the 208 results of Study I samples were classified as outliers as explained below. Therefore only 197 specimens from kidney- (n = 62), heart-

Tab. 3. Analytical recovery of ciclosporin in supplemented whole blood samples

	Day I		Day II		Day I	
Target concentration Ciclosporin (µg/l)	EMIT Ciclosporin (µg/l)	Recovery (%)	EMIT Ciclosporin (µg/l)	Recovery (%)	HPLC Ciclosporin (µg/l)	Recovery (%)
0	<1		<1		<25	
60	64	(107)	62	(103)	65	(108)
70	76	(109)	79	(113)	77	(110)
85	90	(106)	83	(98)	96	(113)
125	119	(95)	116	(93)	130	(104)
175	158	(90)	179	(102)	169	(97)
250	240	(96)	242	(97)	245	(98)
325	283	(87)	337	(104)	318	(98)
375	323	(86)	363	(97)	373	(99)
425	409	(96)	433	(102)	429	(101)

Tab. 4a. Comparisons of the results obtained from whole blood samples from 197 transplant recipients

Group					Regression equation[a]				
	y	vs	x	n	y = ax + b	$\bar{x}$	$\bar{y}$	$s_{y\cdot x}$	r
All	EMIT	vs	HPLC	197	y = 1.09x − 0.6	143.3	155.3[b]	11.0	0.9690
Renal				62	y = 1.12x − 7.4	128.4	135.9[b]	11.5	0.9199
Heart				74	y = 1.06x + 5.8	172.8	189.1[b]	11.8	0.9729
Liver				61	y = 1.10x − 0.7	122.5	134.2[b]	9.2	0.9687
All	RIA	vs	HPLC	197	y = 1.12x − 7.1	143.3	153.3[b]	13.4	0.9570
Renal				62	y = 1.11x − 11.8	128.4	130.5[d]	13.9	0.8819
Heart				74	y = 1.11x − 2.3	172.8	190.0[b]	12.7	0.9716
Liver				61	y = 1.05x + 3.3	122.5	132.5[b]	12.3	0.9395
All	EMIT	vs	RIA	197	y = 0.97x + 6.3	153.3	155.3[d]	13.0	0.9572
Renal				62	y = 1.01x + 4.5	130.5	135.9[c]	13.2	0.8946
Heart				74	y = 0.95x + 8.0	190.0	189.1[d]	13.8	0.9632
Liver				62	y = 1.04x − 4.1	132.5	134.2[d]	12.0	0.9471

Tab. 4b. Comparisons of the results obtained from whole blood samples from 102 paediatric transplant recipients

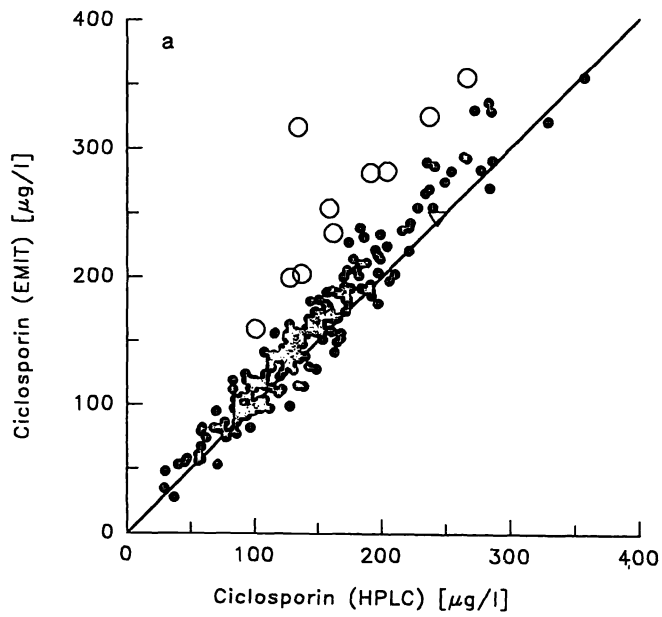
Group					Regression equation[a]				
	y	vs	x	n	y = ax + b	$\bar{x}$	$\bar{y}$	$s_{y\cdot x}$	r
Renal	[2]EMIT	vs	[1]EMIT	102	y = 1.01x − 2.2	118.0	116.5[d]	9.8	0.9575
Renal	[1]EMIT	vs	RIA	102	y = 0.92x + 11.0	116.1	118.0[d]	9.4	0.9608
Renal	[2]EMIT	vs	RIA	102	y = 0.93x + 8.9	116.1	116.5[d]	9.3	0.9621

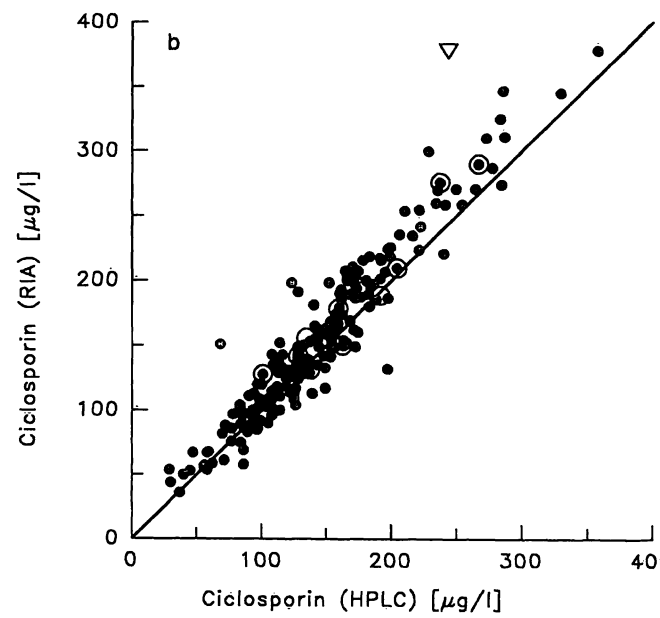
[a] Standardized principal component analysis.
[b,c,d] Significance of the bias $\bar{x} - \bar{y}$ by paired t-test: [b]highly significant ($p < 0.001$), [c]significant ($P < 0.05$), [d]not significant ($P > 0.05$).
[1]EMIT: pretreatment with 100 µl sample volume, double centrifugation, and continuous calibration mode.
[2]EMIT: pretreatment with 200 µl sample volume, single centrifugation, and 2-point calibration mode.

(n = 74), and liver- (n = 61) transplant recipients were evaluated statistically in this study. Good correlations between EMIT, RIA, and HPLC were observed with the ciclosporin results of these remaining 197 samples (tab. 4a). Mean ciclosporin concentrations of EMIT and RIA were 5–10% higher than HPLC results. Ciclosporin measurements by EMIT and RIA did not differ significantly except for the renal transplant group ($P < 0.05$). We observed highly significantly differing results ($P < 0.001$) between ciclosporin determinations obtained by HPLC and by EMIT, and also highly significantly differing results between measurements obtained by HPLC and by RIA with one exception (renal transplant: $P > 0.05$). Ten measurements by EMIT and one by RIA were repeated, because the initial determinations in the excluded eleven specimens were suspected to be wrong. The mean of the residuals (y · x) of the 10 outlying EMIT results was 73.7 µg/l (range: + 53.0 µg/l to + 171 µg/l), while the mean of the residuals of ciclosporin determinations after repeated analysis by EMIT was 10.6 µg/l (range: −3.0 µg/l to + 49 µg/l). The residual of the only outlying RIA result was 112 µg/l, and that of the repeated analysis was 14 µg/l. We decided to exclude the clarified outliers from the statistical evaluation of the correlation study, because the reason for the falsely elevated results could not have been non-specificity. Despite the decision to consider these results outliers the corresponding data points are depicted in figure 2 by extra symbols.

Method comparisons of Study II

The method comparison ^{1}EMIT versus ^{2}EMIT employing two modified assay designs of EMIT yielded





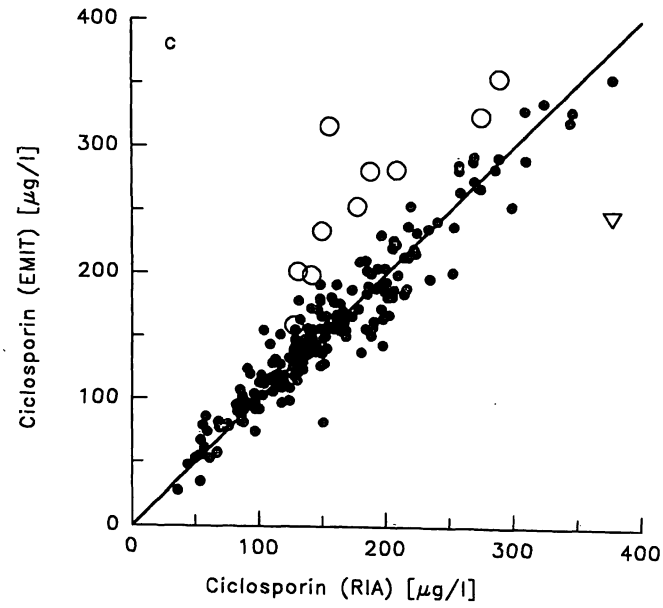


Fig. 2. Study I correlations with 208 ciclosporin concentration measurements in whole blood, and the lines of identity (x = y):
a) EMIT vs HPLC,
b) selective RIA vs HPLC,
c) EMIT vs selective RIA.
Ten results by EMIT (o), and one result by RIA (▽) were classified as outliers. The residuals of these outliers exceeded the corresponding $s_{y\cdot x}$ 5-fold to 15-fold. The data points from repeated analyses were not depicted in the plots. Neither the primary results nor the results obtained by repeated analysis were included in the statistical evaluation of the method comparisons, although the latter fit well into the corresponding correlations.

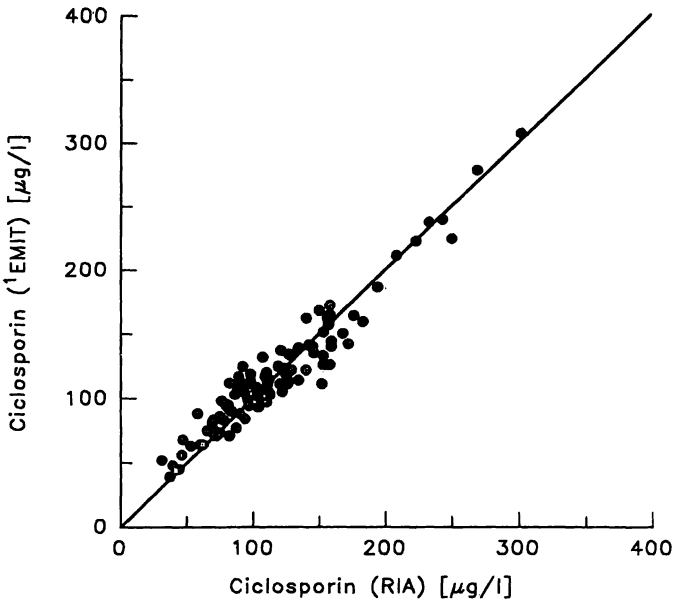


Fig. 3. Study II correlation with 102 ciclosporin concentration measurements in whole blood determined by ^{1}EMIT versus selective RIA, and the line of identity (x = y). The correlation is in good agreement with the corresponding correlation of Study I. However, no outliers were observed in Study II with a modified sample pretreatment.

excellent agreement of the obtained ciclosporin concentration measurements (tab. 4b). We observed outliers neither by sample pretreatment with double centrifugation (^{1}EMIT) nor by sample pretreatment with a double sample volume and only single centrifugation (^{2}EMIT). The two different modes of evaluation of the UV-signals did not cause a significant bias. The correlation studies ^{1}EMIT versus RIA, and ^{2}EMIT versus RIA showed good agreement and had no significant biases (tab. 4b). The plotted ciclosporin concentrations in figure 3 and the statistical data in table 4a/4b revealed that the correlation between the modified EMIT assay and RIA was in good agreement with the results of the corresponding correlation between the original EMIT and RIA (fig. 2).

Discussion

We evaluated the analytical performance of the EMIT for ciclosporin determinations in whole blood. Our method comparison of EMIT versus specific HPLC revealed a significant but small positive bias. The ciclosporin measurements by EMIT averaged 8% higher than those obtained by HPLC. EMIT and RIA agreed very well. Our check of EMIT calibrators revealed a satisfactory agreement between EMIT and HPLC. The data suggested that EMIT appeared to be affected by only minor cross-reactivities of certain ciclosporin metabolites to the same degree as was observed with the selective RIA. This non-specificity of ciclosporin measurements is irrelevant in monitoring transplant patients, and will not influence clinical decisions. Three published studies also revealed satisfactory comparability between ciclosporin measurements by EMIT versus HPLC (14–16). In another study the results obtained by EMIT differed by about 30% from HPLC (17). In this publication the results of the cross-check of EMIT calibrators, and the analytical recoveries in ten specimens provided by Syva were comparable to the results of our study. However, the standard error of estimate obtained from method comparisons with samples from patients was 34.9 μg/l. We observed a standard error of estimate of 11.0 μg/l in our corresponding method comparison with comparable mean ciclosporin concentrations, but ten outlying results had been excluded before the statistical evaluation was performed. Six of the ten excluded ciclosporin results belonged to specimens from renal-, four to specimens from heart-transplant recipients. None of the 10 patients had disturbed liver function or any indication of an accumulation of ciclosporin metabolites. Repeated analysis in these ten specimens did not confirm the ciclosporin values of the original analysis. Outliers were not mentioned in any other publication on the evaluation of the EMIT ciclosporin assay. Other reasons than non-specificity appeared to be responsible for the observed unacceptable inaccuracy. We observed that the sample pretreatment of EMIT occasionally may cause grossly false positive ciclosporin measurements. The source of the observed inadequate accuracy is probably located in the protein precipitation step, and/or the centrifugation procedure. The principle of EMIT limits the selection of highly effective precipitating agents. Heavy metal salts ($CuSO_4$ or $ZnSO_4$) cannot be used because these reagents would deactivate the indicator enzyme glucose-6-phosphate dehydrogenase. It was to be expected that a mixture of sample and methanol (1 volume to 2 volumes) would not form a solid pellet of precipitated proteins after centrifugation. The protein pellet tended to release floating particles. We supposed that in some samples turbidity, even though not visible to the eye, was the explanation for poorly reproducible and incorrect results. We obtained satisfactory accuracy and reproducibility when the supernatant was centrifuged twice. Alternatively, the use of a larger sample volume for the sample pretreatment seemed to be similarly effective, and less labour-intensive. Both modifications required very careful handling of the sample cups after centrifugation, and during uptake of the supernatant. The results of our method comparison Study II comparing two modifications of sample pretreatment (^{1}EMIT vs ^{2}EMIT) showed that both sample pretreatment procedures were able to effectively avoid outlying measurements. Different

calibration modes had no influence on the results of Study II with samples from patients. However, our studies of the precision of EMIT indicated that calibration and ciclosporin measurements by use of the 2-point mode were able to improve the assay precision, especially for the determination of ciclosporin concentrations below 100 µg/l. This finding might be of use if ciclosporin monitoring in a low concentration range is required. At present we recommend 2-point calibration to achieve a lower imprecision of measurement by EMIT. Correctly, Syva's recommendation to users of the assay is to prolong the measuring time from 350 s to 550 s and to record eleven signals instead of four [personal communication from Syva, Darmstadt, Germany]. The random variation of the determined mean absorbance difference averaged over 11 intervals should be lower. Consequently, an improved assay precision should be obtainable by means of this modified measuring design.

In conclusion, the EMIT™ cyclosporine assay provides highly specific measurements of ciclosporin in whole blood. The precision of the EMIT at ciclosporin concentrations > 100 µg/l is adequate. For improved precision of ciclosporin concentration measurements below 100 µg/l the assay should be performed using the described 2-point measuring design. Ciclosporin analysis can be completed after 1–2 hours. The accuracy of the assay depends on skilful analytical performance by well-trained personnel. However, repeated analysis may be necessary if the plausibility of ciclosporin measurement in a follow-up of ciclosporin monitoring is questionable. The EMIT assay is well suited for regular ciclosporin monitoring.

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